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# A One-Pot Chemically Cleavable Bis-Linker Tether Strategy for the Synthesis of Heterodimeric Peptides

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***Abstract:*** *Heterodimeric peptides linked by disulfide bonds are attractive drug targets. However, their chemical assembly can be tedious, time-consuming, and low yielding. Inspired by the cellular synthesis of pro-insulin in which the two constituent peptide chains are expressed as a single-chain precursor separated by a connecting C-peptide, we have developed a novel chemically cleavable bis-linker tether which allows the convenient assembly of two peptide chains as a single "pro"-peptide on the same solid support. Following the peptide cleavage and post-synthetic modifications, this bis-linker tether can be removed in one-step by chemical means. This method was used to synthesize a drug delivery-cargo conjugate, TAT-PKCi peptide, and a two-disulfide bridged heterodimeric peptide, thionin (7-19)-(24-32R), a thionin analogue. To our knowledge, this is the first report of a one-pot chemically cleavable bis-linker strategy for the facile synthesis of cross-bridged two-chain peptides.*

Cystine-rich heterodimeric peptides or small proteins, such as insulin and relaxin, are attractive targets for development of next-generation therapeutics owing to their pleiotropic physiological roles.[1] Structure–function relationship studies of these hetero-dimeric peptides play a crucial role in the development of new lead compounds. Their chemical synthesis can be achieved by, for example, separate assembly of the individual S-reduced chains and subsequent oxidative folding. However, modified analogues often cannot spontaneously fold because of loss of secondary structure within the chains. Consequently, directed, stepwise disulfide bridge formation is performed.[2] This requires orthogonal S-protection and necessitates solid-phase synthesis of the two separate chains, followed by their release from the solid support and multistep solution-phase reactions to control the correct disulfide pairing leading to lower yields.[3] While this method can be very effective, its complexity emphasizes the need for an improved synthetic route to heterodimeric peptides whereby the number of synthetic steps is reduced.

The in vivo cellular production of insulin has a C-peptide that is used as a temporary link between the B- and A-chains. Once the precursor pro-peptide is folded, the C-peptide is enzymatically cleaved to generate the final native two-chain peptide. We thus developed an analogous method whereby both chains are sequentially synthesized on the same solid support separated by a chemically cleavable bis-linker tether (Figure 1). After isolation of the single chain and disulfide folding, the surrogate C-peptide tether is efficiently removed. Similar elegant strategies for biomimetic insulin syntheses have been developed over the years.[4] However, these approaches are limited by the need for enzyme(s) for release of the C-peptide mimic or use of specific residues (e.g. Thr and Glu) at particular positions within the individual peptide chains. To overcome these limitations, we have developed a bis-linker tether which mimics the role of a C-peptide (Figure 1). This bis-linker tether consists of a small peptide with a chemical spacer placed between two chemical moieties, one an N-terminal and the other a C-terminal linker. Once the entire chain assembly is accomplished, the linear two-chain peptide can be folded or conjugated by chemical means,

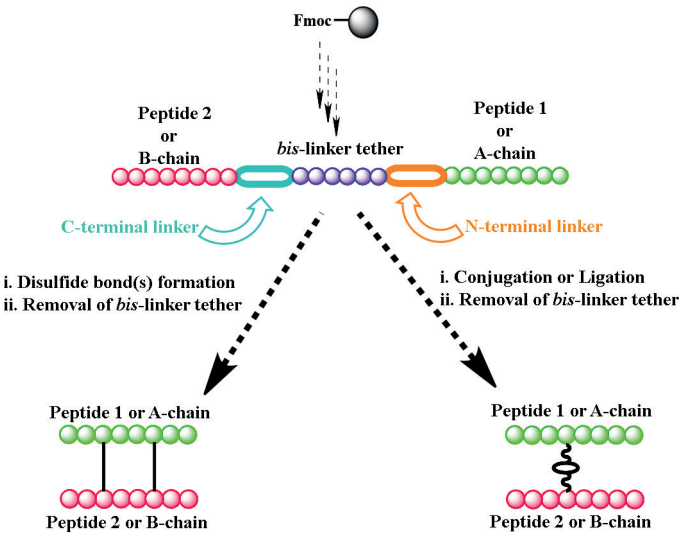


***Figure 1.*** The proposed strategy for the synthesis of covalently inter-linked two-chain peptides.

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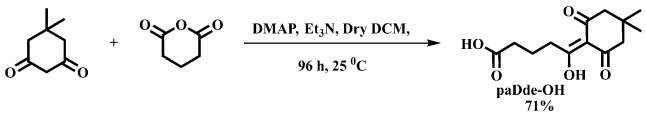
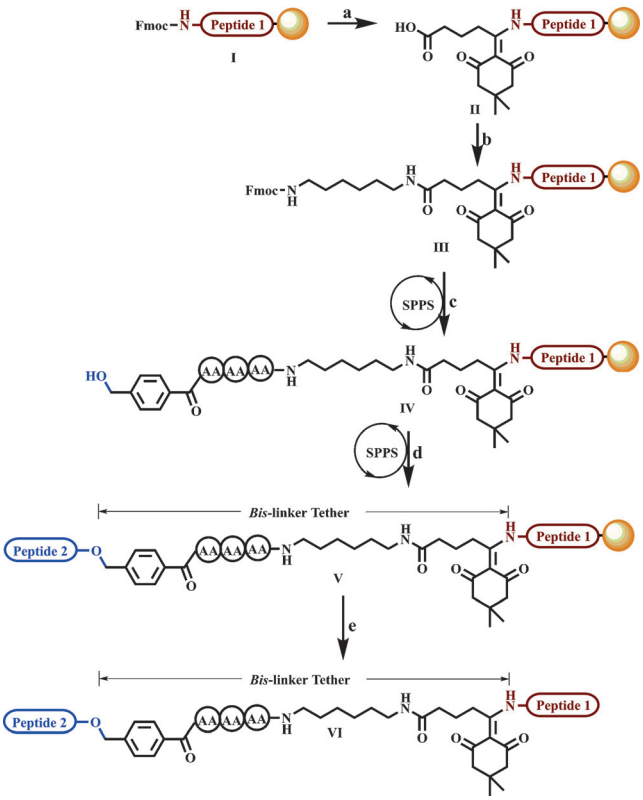
followed by removal of the bis-linker tether (Figure 1). Importantly, the two chemical linkers must enable the generation of native free N- and C-termini which is often essential for the activity of some native two-chain peptides.[5] Another important advantage of having separate N- and C-terminal linkers is the flexibility of choosing the length and nature of the intermediate tether sequence that can be used to mimic the C-peptide.

The proposed strategy (Figure 1) requires that the two chemical linkers are stable to Fmoc-solid phase peptide synthesis (SPPS), acidic peptide cleavage, the removal of the thiol protecting groups, and disulfide bond forming conditions (thiolysis). The use of hydroxymethylbenzoic acid (HMBA) as a chemically cleavable C-terminal linker has previously been demonstrated.[6] HMBA is also compatible with various thiol protecting groups as it can be easily hydrolyzed at basic pH. An ideal N-terminal linker (e.g. on peptide 1; Figure 1) should have these characteristics and be simultaneously removable with the C-terminal HMBA linker. However, to our knowledge, no such linker has been reported to date. Several amino protecting groups have been developed[7] including the dimedone-based amino protecting groups 1-(4,4-dimethyl-2,6-dioxocyclohexyli-dene)ethyl (Dde) or 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde) which have been used in solid-phase peptide chemistry owing to their orthogonality to Fmoc- and Boc-SPPS[8] and, thus, provide a suitable basis for its development into an N-terminal linker. Moreover, the dimedone-based protecting group can be readily coupled onto a free amine and efficiently removed in the presence of dilute aqueous hydrazine solution. Another major advantage of hydrazine-aided removal of the dimedone protecting group is that an additional scavenger is not required as its removal results in the concomitant formation of a non-reactive indazole ring structure.[9] Existing disulfide bonds are also reported to be stable under hydrazinolysis conditions.[10] Importantly, the basic pH of the hydrazine buffer also allows the simultaneous one-pot removal of the HMBA linker that serves as a C-terminal linker in our strategy.[7,11] Thus, a modified form of the ivDde amino protecting group, 5-(4,4-dimethyl-2,6-dioxocyclohexylidene)-5-hydroxypentanoic acid (paDde-OH, Scheme 1), was synthesized as a novel N-terminal cleavable




***Scheme 1.*** Synthesis of dimedone-based N-terminal linker, paDde-OH.

linker. The Knoevenagel condensation of 5,5′-dimethylcyclohexane-1,3-dione with the respective cyclic anhydride was advantageous over the reported two-step synthesis that employs a synthesis of tertiary butyl monoester of dicarboxylic acid which is then converted into the desired products.[12] Our one-step synthesis method from glutaric anhydride yielded the desired N-terminal linker "paDde-OH" (Scheme 1) in a yield of 71% (Supporting Information, Section 3).




***Scheme 2.*** Strategy for solid-phase peptide synthesis (SPPS) of two peptide chains tethered by a bis-linker sequence: a) i) 20% piperidine/DMF, ii) paDde-OH, DIEA, DMF; b) i) HCTU (2.5 equiv.), DIEA (2.5 equiv.), *N*-Fmoc-1,6-diaminohexane (2.5 equiv.); c) i) SPPS, ii) 4-hydroxymethylbenzoic acid (4 equiv.), HCTU (3.8 equiv.), DIEA (4 equiv.), DMF; d) i) coupling of the first amino acid of peptide 2 (Fmoc-amino acid-OH (4 equiv.), DIC (2 equiv.), DMAP (0.1 equiv.), DMF); ii) SPPS and e) cleavage of the linear peptide from solid support (TFA:anisole:DODT:TIPS (94:3:2:1)).

The first step in the overall method was to sequentially assemble both peptides on a single solid support. A standard Fmoc-SPPS was employed to assemble peptide 1 (Scheme 2, **I**). The N-terminal linker, paDde-OH (obtained from Scheme 1) was coupled onto the free α-amine of the N-terminal amino acid of peptide 1 in the presence of *N*,*N*-diisopropylethylamine (DIEA) which results in a free carboxylic acid group on the resin-bound peptide (**II**). This was activated with *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(6-chloro-1*H*-benzotriazol-1-yl)uranium hexafluorophosphate (HCTU)/DIEA followed by coupling of a Fmoc-mono-protected diamine to yield resin-bound Fmoc-protected peptide (**III**). Further, standard stepwise solid-phase couplings can be continued to assemble the tether including the HMBA linker to obtain a free hydroxyl group on the resin-bound peptide (**IV**). To assemble the second peptide, the C-terminal amino acid of peptide 2 was anchored using *N*,*N*′-diisopropylcarbodiimide (DIC)/ *N*,*N*-dimethylamnopyridine (DMAP)-mediated acylation on HMBA, followed by standard SPPS amino acid couplings to complete the second sequence (**V**). Finally, the resin-bound tethered two-peptide product was cleaved using a TFA cleavage cocktail (**VI**).

To evaluate the proposed bis-linker tether strategy, a suitable two-peptide system (TAT-PKCi) was selected as a proof of concept study. Transactivator of transcription (TAT) is a cell penetrating peptide sequence that has been utilized to deliver a therapeutically important phosphokinase C inhibitor (PKCi).[13] PKCi as a first peptide, TAT as second peptide and HMBA-Gly-diaminohexane-paDde as the bis-linker tether were assembled using the general solid-phase method in Scheme 2 (Figure 2 A). The resulting linear peptide, PKCi-bis-linker-TAT (**2A**, Figure 2 A) was then used as a starting material for the formation of the intra-peptide-peptide conjugate. This was obtained in a one-pot fashion (either by disulfide or thioether bond) followed by removal of the bis-linker tether (Figure 2 A). The disulfide bond was formed using 2,2′-dithiodipyridine (DPDS) oxidation that resulted in a cyclic PKCi-bis-linker tether-TAT peptide (**2B**).[14] In addition, the linear PKCi-bis-linker tether-TAT peptide (**2A**) was treated with α,α′-bis-bromo-*m*-xylene which resulted in a cyclic two-peptide product (**2E**, Figure 2 A) to further investigate the compatibility of this linker

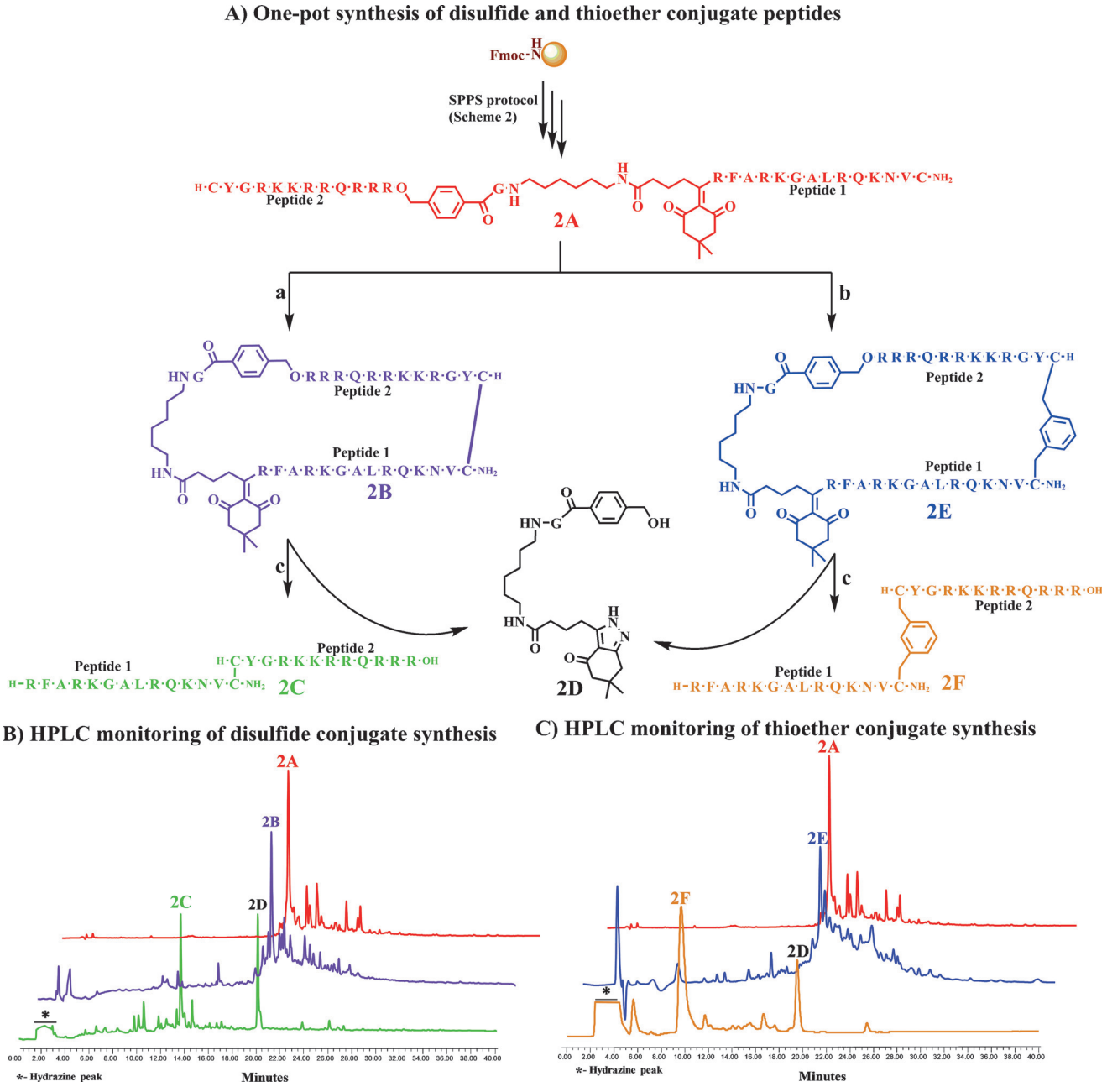



***Figure 2.*** A) One-pot conjugation and removal of bis-linker tether a) 1.5 equiv. DPDS, acetonitrile: water; b) α,α′-bis-bromo-*m*-xylene, phosphate buffer, pH 7.4; c) 5% hydrazine buffer. B) RP-HPLC monitoring of crude peptides (gradient 10–50% buffer B, 40 min, 1% $min^{-1}$ flow rate.): linear PKCi-bis-linker tether-TAT, **2A**; cyclic PKCi-bis-linker tether-TAT disulfide conjugate, **2B**; PKCi-TAT disulfide conjugate, **2C**; cleaved bis-linker tether, **2D**; and C) RP-HPLC monitoring for crude peptides (gradient 10–50% buffer B, 40 min, 1% $min^{-1}$ flow rate): cyclic PKCi-bis-linker tether-TAT thioether conjugate, **2E**; PKCi-TAT thioether conjugate, **2F**.

with non-disulfide conjugation strategies.[15] Given the hydrophobic nature of both the bis-linker tether and the xylene moiety, the RP-HPLC profile of the cyclic peptide (**2E**) was found to be broad (Figure 2C). However, the addition of 5% aqueous hydrazine for 5 min. produced the desired PKCi-TAT conjugates (**2C** and **2F**) with sharp HPLC peaks and liberated the bis-linker tether (**2D**) without forming acylhydrazine (Supporting Information).[16] Reported methods for making this peptide–peptide conjugate involve two separate syntheses of individual peptide chains. Our one-pot reaction reduced the number of steps and purifications to yield the desired product (**2C**, Figure 2B) with an overall yield of 27% and the desired PKCi-TAT thioether conjugate (**2F**, Figure 2C) obtained in 21% overall yield (calculated from crude **2A**).

Finally, to evaluate the proposed bis-linker tether strategy, the synthesis of a more complex model peptide, thionin (7-19)-(24-32R), a thionin analogue, with two chains and two disulfide bonds was attempted (Figure 3).[17] Both cystine residues can be formed regioselectively using trityl (Trt) and acetamidomethyl (Acm) thiol protecting groups (Figure 3A). Compared with peptide, such as insulin, this thionin analogue is a relatively small and hydrophilic sequence and thus an ideal model system to study the compatibility of the bis-linker tether approach together with regioselective disulfide bond formation. Using the same general method of synthesizing two peptides on a single solid support (Scheme 2), both the A- and B-chains were assembled, with an additional Lys and Arg in the tether to provide enough length to form the disulfide bonds and also to avoid the aggregation that we observed in the synthesis of peptide **2E**. The linear A-chain-bis-linker-B-chain peptide (**3A**) was subjected to simple DPDS-mediated oxidation to form the first disulfide bond (**3B**). After iodine-mediated removal of Acm groups and simultaneous second disulfide bond formation to obtain **3C**, the desired peptide (**3D**) was obtained by the addition of aqueous hydrazine in an overall yield of 24% as calculated from crude **3A**. Notably, epimerization of Fmoc-AA-OH may take place, especially in case of esterification involving Cys and His.[18] We did not observe such epimerization as Arg is the first amino acid of peptide 2, in both PKCi-TAT (Figure 2) and thionin analogue (Figure 3). Epimerization of Cys or His can also be avoided as reported by Sandhya and Ravindranath.[18]

In conclusion, a novel dimedone-based linker was developed and used together with an HMBA-based linker to synthesize a heterodimeric peptide (thionin analogue) as well as peptide–peptide conjugates (PKCi-TAT). Importantly, this bis-linker tether method is compatible with both Fmoc- or Boc-SPPS. Since this strategy reduced the number of intermediate and purification steps, the peptides were obtained in good overall yields. This preliminary study on the stability of the bis-linker and its removal followed by regioselective disulfide bridge formation will enable the preparation of two chain cysteine-rich peptides and proteins, such as insulin and insulin-like peptides (e.g. relaxin). Since dimedone-based protecting groups are widely used in solid-phase synthesis including of peptide nucleic acids and in carbohydrate chemistry, this linker could also be applicable to the synthesis of multiple polynucleotide or polysaccharide chains.

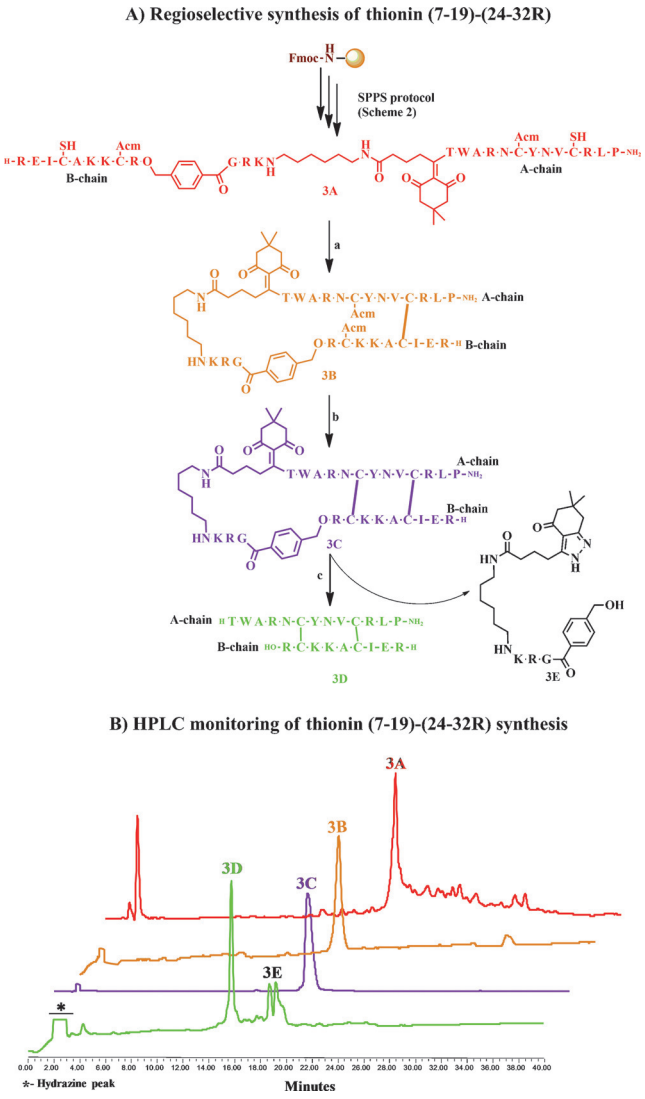



***Figure 3.*** A) Regioselective approach for the synthesis of thionin analogue: a) 1.5 equiv. DPDS, acetonitrile:water, b) 60 mM iodine solution in acetic acid, c) 5% hydrazine buffer. B) RP-HPLC monitoring of peptides (gradient 10–50% buffer B, 40 min, 1% $min^{-1}$ flow rate.): crude reduced thionin analogue (A-bis-linker tether-B), **3A**; purified oxidized thionin analogue (A-bis-linker tether-B)- one disulfide bond, **3B**; purified oxidized thionin analogue (A-bis-linker tether-B) with two disulfide bonds, **3C**; crude thionin analogue (A-B), **3D**; and cleaved bis-linker tether, **3E**.

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